

# CONFORMATION STUDIES WITH POLYPEPTIDES BY ROTATORY DISPERSION AND THIN-FILM DIALYSIS\*

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Following earlier attempts by many investigators to correlate specific types of rotatory dispersion curves with particular arrangements in polymeric macromolecules, the idea developed that "anomalous" curves<sup>1</sup> were significant with regard to possible helical structure. Moffitt and Yang<sup>2</sup> proposed a treatment of the dispersion data then available by means of an equation requiring characteristic constants. It was thought that a high negative value for one of these constants,  $b_0$ , was an indication of helical structure. When spectropolarimeters capable of measuring rotations at shorter wavelengths became available, Simmons and Blout<sup>3</sup> observed a striking type of rotatory dispersion with polyamino acids and proteins thought to have a helical structure. A strong Cotton effect was observed in the 220-m $\mu$  region, with a deep negative minimum at about 233 m $\mu$ .

These observations were seized upon by protein chemists and molecular biologists as a means of demonstrating conformational structure and even of calculating the per cent of helical content from the depth of the minimum at 233 m $\mu$ . From a theoretical and practical standpoint, the idea seemed very attractive, and with the recent availability of spectropolarimeters capable of making accurate measurements at these low wavelengths in spite of the relatively high absorption of light by the biopolymers, the literature soon became flooded with studies of supposed helical structures based on rotatory dispersion measurements.

About three years ago, it was observed in this laboratory<sup>4</sup> that the cyclic decapeptides gramicidin S-A and tyrocidine-B both gave the characteristic rotatory dispersion curves shown in Figure 1. Both are cyclic decapeptides whose covalent structures are known,<sup>5-7</sup> but are such as to make very unlikely the formation of an undistorted helical structure. The warning that these observations implied appears to have been disregarded, if not overlooked, and many studies continued to appear in which the type of rotatory dispersion shown in Figure 1 was equated with a helical conformation. A survey of other cyclic peptides therefore seemed of interest.

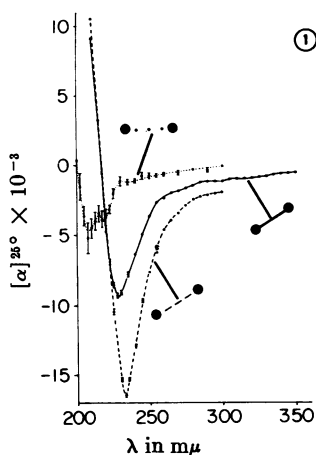


FIG. 1.—(●—●) Gramicidin S-A in H<sub>2</sub>O; (●—●) tyrocidine B in H<sub>2</sub>O; (●...●) tyrocidine B with the ring split.

**Methods.**—Gramicidin S-A was prepared by counter-current distribution (CCD) as reported earlier.<sup>8</sup> The tyrocidines were also prepared by CCD<sup>7</sup> from a sample supplied by the Wallerstein Company. Gramicidin A was prepared by CCD<sup>8</sup> from the crude crystal-

line material also furnished by the Wallerstein Company. Bacitracin A was prepared by CCD by a procedure soon to be described.<sup>9</sup> The crude material was a gift from the Commercial Solvents Company. Polymyxin B<sub>1</sub> was prepared by CCD<sup>10</sup> from a crude crystalline sample furnished by the Pfizer Company. The adrenocorticotropin (ACTH) was prepared by CCD<sup>11</sup> from a sample of crude hog ACTH furnished by Dr. Hays of the Riker Laboratories. The isoasparagine angiotensin was a gift from Dr. Schwyzer (Ciba). We thank Dr. Hays and Dr. Schwyzer and the various companies for these materials.

L-Tyrosine diketopiperazine and L-tyrosyl-L-tyrosine were purchased from Cyclo, ergotamine from Sigma Chemical Company.

The rotatory dispersion measurements were made in a Cary 60 spectropolarimeter. The results are expressed as plots of specific rotation against wavelength. Thin-film dialysis experiments were performed as previously described.<sup>12</sup>

**Results.**—It was found that tyrocidines A and C both gave essentially the same type of rotatory dispersion as that shown in Figure 1 for tyrocidine B. On the other hand, bacitracin A<sup>13</sup> gave a more complicated type of dispersion as shown in Figure 2. A transformation form of bacitracin A called bacitracin F<sup>14</sup> gave the slightly modified curve, and the isomeric forms of oxidized bacitracin A<sup>15</sup> gave the simpler dispersion curves shown in Figure 2.

The diketopiperazine of L-tyrosine gave the striking curve shown in Figure 3 as compared to that of L-tyrosyl-L-tyrosine. The cyclic antibiotic polymyxin B<sub>1</sub> gave the dispersion curve shown in Figure 4, which also shows the type of curve given by the condensed tetrapeptide, ergotamine.<sup>16</sup>

A number of linear peptides also were studied. These included ACTH in 0.01 *N* acetic acid and at pH 10.92 (Fig. 5) and the isoasparagine of angiotensin (Fig. 6). A number of peptides isolated from the tryptic digestion of the alpha and beta chains of hemoglobin were likewise studied.<sup>17, 18</sup> These included T $\alpha$ 9, T $\alpha$ 12, and T $\beta$ 12, whose sequences and dispersions are shown in Figure 7.

In Figure 8 is shown the dispersion curve of gramicidin A in methanol. From the work of Sarges and Witkop,<sup>19</sup> it is supposed to be a linear peptide.

**Discussion.**—After our publication,<sup>4</sup> Balasubramanian and Wetlaufer<sup>21, 22</sup> investigated the behavior of the smallest possible peptide-ring system, the diketopiperazines, and found that the diketopiperazine of L-alanyl-L-serine showed a monotonic curve similar to that found for tyrocidine B and gramicidin S-A. The diketopiperazine of L-tyrosyl-L-tyrosine gives a deeper minimum (Fig. 3).

Another cyclic peptide, bacitracin A (Formula 2), in water gave the interesting curve shown in Figure 2. The minimum at 230  $\mu$  was only about one tenth as deep as that for L-tyrosyldiketopiperazine or gramicidin S-A. Bacitracin A has two ring structures, one containing six or seven (most probably seven) amino acid residues<sup>23</sup> and the other a much smaller thiazoline ring system. The maximum at 270  $\mu$  relates to the thiazoline ring, since splitting this ring by oxidation gives a substance that shows the monotonic curve with the minimum at 230  $\mu$ . Partial modification of the thiazoline ring by oxidation to the thiazole bacitracin F<sup>14</sup> modifies the positive maximum of bacitracin A but does not eliminate it. This is interesting because this transformation eliminates the optical activity of the  $\alpha$  carbons of both the terminal isoleucine and cysteine residues. Both types of oxidation decrease the depth of the minimum at 230  $\mu$  as might be expected if there is an interaction between the N-terminal part of the molecule and the phenylalanine as postulated.<sup>13</sup>

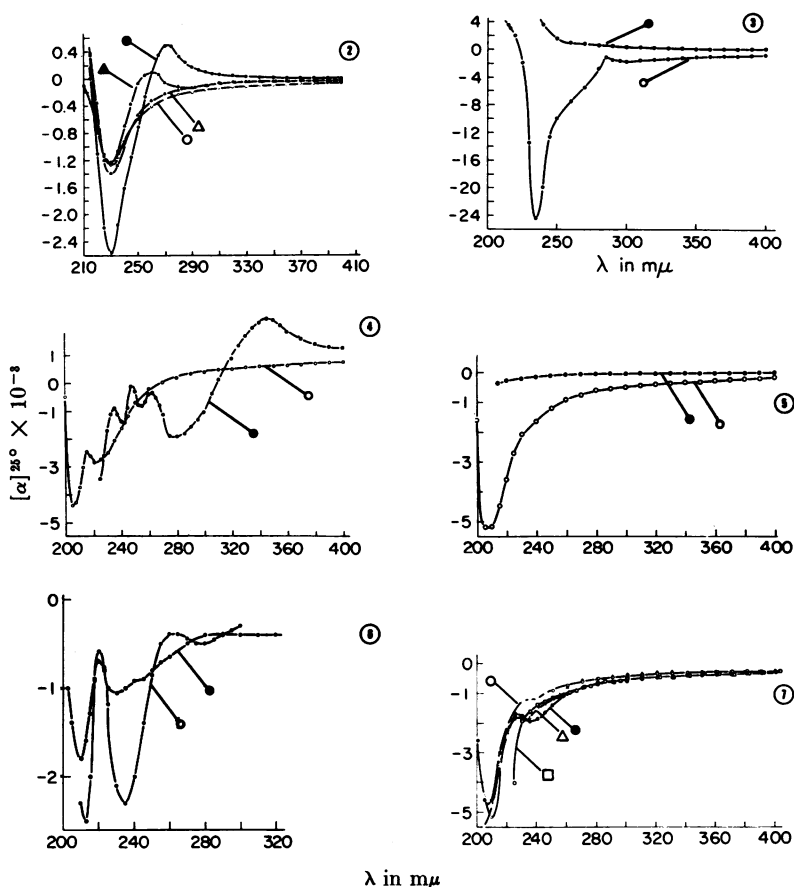


FIG. 2.—(●—●) Bacitracin A in H<sub>2</sub>O; (▲—▲) bacitracin F in H<sub>2</sub>O; (○—○) oxidized allo-bacitracin A in H<sub>2</sub>O; (△—△) oxidized bacitracin A in H<sub>2</sub>O.

FIG. 3.—(○—○) L-tyrosine diketopiperazine in H<sub>2</sub>O; (●—●) L-tyrosyl-L-tyrosine diketopiperazine in H<sub>2</sub>O.

FIG. 4.—(○—○) Polymyxin B<sub>1</sub> in H<sub>2</sub>O; (●—●) ergotamine in methanol.

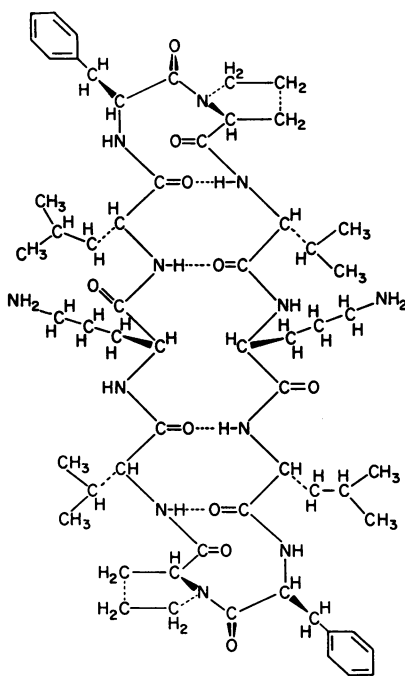
FIG. 5.—(○—○) ACTH in 0.01 *N* acetic acid; (●—●) ACTH in H<sub>2</sub>O at pH 10.92.

FIG. 6.—Isoasparagine angiotensin. (●—●) pH 5.67; (○—○) pH 10.95.

FIG. 7.—(●—●) Tα<sub>9</sub>; Val-Asp-Pro-Val-Asp(NH<sub>2</sub>)-Phe-Lys in 0.01 *N* acetic acid. (○—○) Tα<sub>12</sub>; Val-Gly-Ala-His-Ala-Gly-Glu-Tyr-Gly-Ala-Glu-Ala-Leu-Glu-Arg in 0.01 *N* acetic acid. (△—△) Tα<sub>12</sub> at pH 10.95. (□—□) Tβ<sub>12</sub>; Leu-Leu-Val-Val-Tyr-Pro-Tyr-Thr-Glu(NH<sub>2</sub>)-Arg in 0.01 *N* acetic acid.

In view of this behavior, it is of interest to test the rigidity of these substances by thin-film dialysis.<sup>24</sup> It was found that the dialysis rate of gramicidin S-A was not influenced by the addition of methanol to the solvent in contrast to bacitracin A and polymyxin B (Formula 3). Nuclear magnetic resonance studies<sup>25</sup> of deuterium exchange and tritium exchange<sup>26</sup> indicated four slowly exchangeable protons with gramicidin S-A, but tritium exchange indicated only one for bacitracin. Gramicidin S-A must, therefore, be a relatively rigid structure. Many conformations have been proposed for gramicidin S-A.<sup>27-34</sup> Formula 1, sug-

FORMULA 1.—A hypothetical structural formula of gramicidin S-A.

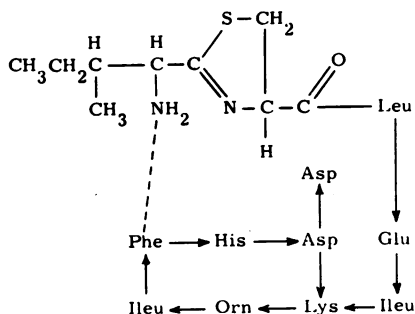


gested in part by Hodgkin and Oughton<sup>27</sup> and by Schwyzer,<sup>29</sup> is the only one consistent with our data.<sup>25, 26</sup>

ACTH is a peptide hormone of 39 amino acid residues with no covalent cross-links and is thought to be a random coil.<sup>35</sup> With the thin-film dialysis technique, its rate of dialysis in 0.01 *N* acetic acid is slowed<sup>11</sup> from a half-escape time of 1 hour to 13 hours by the addition of ammonium acetate (0.15 *M*), indicating a major change in shape. Association was eliminated as a possible cause by ultracentrifuge studies. Optical rotatory dispersion (ORD) measurements in 0.01 *N* acetic acid gave the curve shown in Figure 5, with the deep minimum at 207  $\mu$ . Since ACTH does not dialyze at a high pH range, the ORD curve was determined at pH 10.92. A major shift from that at pH 3.4 was noted. Addition of ammonium acetate to the 0.01 *N* acetic acid solution caused no shift in the ORD curve.

This result contrasts sharply with that found for the linear octapeptide, is-asparagine angiotensin. Here again, the dialysis rate was markedly slowed<sup>20</sup>

FORMULA 2.—A partial hypothetical formula of bacitracin A.





of ammonium acetate as contrasted to T $\alpha$ 12 and T $\beta$ 12. T $\beta$ 12 showed no change in shape upon addition of ammonium acetate with the dialysis technique in contrast to the others.

From the data on these and a number of other peptides, both linear and cyclic, it would appear that ORD data alone cannot be interpreted reliably in terms of any one type of conformation. A striking type of Cotton effect apparently is much more likely with a small fixed ring structure. Probably a simple diketopiperazine will always give a strong minimum at about 233 m $\mu$  because of its rigid nature, but larger ring structures may or may not do so as evidenced by polymyxin (Fig. 4), or cyclotetraalanine.<sup>22</sup> It is of some interest that ergot alkaloids, which were shown by Jacobs and Craig<sup>37</sup> to be cyclic tetrapeptides and later postulated by Stoll, Hofmann, and Petrzilka<sup>38</sup> to be cyclol or transannular structures, give a Cotton effect with the minimum, as shown by ergotamine (Fig. 4), at a much longer wavelength. The displacement of the effect may be influenced by the neighboring groups and the replacement of an amide group by an ester group.

The cyclic polypeptide antibiotics are more likely to have small incipient ring structures, perhaps tautomeric and not entirely covalently held, which could give the effect. Even so-called linear peptides may show the effect in a specific solvent environment as shown by Schellman and Nielsen.<sup>39</sup> Gramicidin A, thought to be linear<sup>19</sup> and to have 15 amino acid residues, gave in methanol the ORD curve shown in Figure 8. The gramicidins have been postulated to be cyclol<sup>40</sup> derivatives.<sup>44</sup> However, even the evidence for the case most acceptable, that of the ergot alkaloids, was based on alkaline reduction.<sup>38</sup> Actually, the key to the nature of the ergot alkaloids was obtained 35 years ago by a sodium-butanol reduction<sup>41</sup> and isolation of the resulting piperazines. Perhaps one of the so-called structures revealed by ORD is a relatively rigid structure that can easily go over to a diketopiperazine or cyclol structure in alkaline solution. The Phe-Pro sequence might be suspect in this connection since peptides of proline are known to form diketopiperazines with great ease<sup>42</sup> and Phe-Pro gave trouble in its determination in tyrocidine B' due to cyclization. The Phe-Pro linkage is present in certain ergot alkaloids, and proline in others.

Gramicidin S-A has two such linkages but the tyrocidines have only one. Experimentally, the minimum at 233 m $\mu$  in gramicidin S-A is twice as strong as in the tyrocidines (Fig. 1). A relatively rigid ring structure could be responsible for the type of rotatory dispersion noted. Gramicidin S-A has two or four protons, depending on the pH, which are very slow to exchange with tritium as will be reported later.<sup>25, 26</sup> Transannular interaction with smaller ring compounds has been shown<sup>43</sup> to give rise to strong Cotton effects even when covalent bonding across the ring was lacking. A transannular interaction involving two peptide

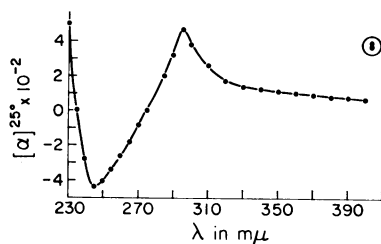


FIG. 8.—Gramicidin A in methanol.

linkages has never been clearly demonstrated but may require a special rigidity in a favorable spacial arrangement.

The experience gained in this study indicates that the type of rotatory dispersion widely ascribed to a helical conformation in macromolecules may also arise from other conformations, perhaps even a rather local rigidity in the molecule.

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